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(54) Title: ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS

(57) Abstract

The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been demonstrated to include a region which results in selective expression in endothelial cells, between -1 and -220 based on the positions relative to the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expresion in large vessel endothelial cells, as opposed to all endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin or serum or to effect selective expression in endothelial cells generally or preferentially in endothelial cells of the large blood vessels. The gene control elements include elements responsive to environmental stiumuli (either thrombin or serum); and information to determine distribution of the desired protein expression (large vessels). Therapeutic strategies include the use of the minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of gene therapy where systemic distribution is desired; the use of a promoter including an environmental stimuli response element(s), for use in delivery of agents whose expression should be increased during times of increased thrombin/platelet activation or during regional trauma; the use of the minimal promoter including an environmental stimuli response element and the element directing expression to large vessel endothelium, where a response to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal promoter and element directing expression to large vessel endothelium, where expresion is specifically targeted to large vessel endothelium but is not increased in response to any particular stimuli.

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ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS

Background of the Invention

The United States government has certain rights in this invention by virtue of National Heart, Lung and Blood Institute of the Institutes of Health grant No. P01 HL54804 to Charles T. Esmon.

The present invention is generally in the area of targeting and regulation of expression of recombinant gene constructs incorporating regulatory elements present in the promoter of an endothelial cell protein C/activated protein C receptor.

Atherosclerosis and most other vascular disease primarily occur in large vessels. Endothelial cells are a primary defense mechanism against cellular infiltration and thrombosis. Abnormal function of the endothelial cells contribute to myocardial infarction (MI), stroke and the development of atherosclerotic plaque. The delivery of proteins or protein expression inhibitors, directly or via gene therapy, specific to large vessel endothelial cells, is one means for addressing these clinical conditions. For example, the anti-thrombotic potential of endothelium can be increased by delivering agents that prevent thrombosis, such as thrombomodulin, heparin proteoglycans, tissue factor pathway inhibitor (TFPI, a potent inhibitor of the tissue Factor-Factor VIIa-Factor Xa complex), etc. Fibrinolytic activity can be increased by overexpression of tissue plasminogen activator (tPA) or urokinase. Expression of adhesion molecules such as P-selectin or ICAMs can be inhibited to minimize or decrease the probability of atherosclerotic plaque rupture.

Targeting endothelial cells non-specifically is often inadequate. Since more than 95% of endothelial cells are in the capillaries, any therapy directed toward endothelial cells per se runs the risk of systemic complications. One must be confident that the gene expression is limited to the desired cells when using a gene therapy approach.

It is therefore an object of the present invention to provide means and methods for selective expression of genes, especially in endothelial cells, and even more specifically in large vessel endothelial cells.

It is a further object of the present invention to provide means and methods for selective expression of genes in response to specific stimuli.

Summary of the Invention

The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been demonstrated to include a region which results in selective expression in endothelial cells, between -1 and -220 based on the positions relative to the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expression in large vessel endothelial cells, as opposed to all endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1).

The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin or serum or to effect selective expression in endothelial cells generally or preferentially in endothelial cells of the large blood vessels. The gene control elements include elements responsive to environmental stimuli (either thrombin or serum); and information to determine distribution of the desired protein expression (large vessels). Therapeutic strategies include the use of the minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of gene therapy where

systemic distribution is desired: the use of a promoter including an environmental stimuli response element(s), for use in delivery of agents whose expression should be increased during times of increased thrombin/platelet activation or during regional trauma; the use of the minimal promoter including an environmental stimuli response element and the element directing expression to large vessel endothelium, where a response to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal promoter and element directing expression to large vessel endothelium, where expression is specifically targeted to large vessel endothelium but is not increased in response to any particular stimuli.

Brief Description of the Drawing

Figure 1 is a comparison of the nucleotide sequences of the murine EPCR and human EPCR promoters (SEQ. ID Nos. 1 and 2, respectively).

Figure 2 is a graph of relative levels of expression (relative luminescent units) for mP3340, mP1120, mP700, mP350 and control SV40, in bovine endothelial cells (large vessel endothelial cells), rat heart endothelial cells, mostly capillary cells (small vessel endothelial cells), and 293 kidney cells (non-endothelial cells).

Figure 3 is a schematic of the constructs transfected into bovine aortic (large vessel) endothelial cells, graphing the relative levels of expression (relative luminescent units) for mP1120, mP550, mP350 (AP1 mutant), mP350 (deletion from 280 to 160), mP280, mP220, mP180, mP160, and mP80, with the pGL3 control.

Figure 4 is a schematic of the promoter. The top line indicates the structure of the promoter from -220 to -180, which includes the transcription control elements required for constitutive expression in endothelial cells. AP-4 and SP-1 are known promoter elements that bind proteins that control gene expression. The bottom line is a schematic representation of the EPCR promoter showing the locations of the large

vessel specific element between -1080 and -700 ("C"), the element which includes the sequence responsible for thrombin induction ("B"), the endothelial specific region ("A"), and the EPCR encoding element. SP-1, AP-1 and AP-4 are known promoter elements which bind proteins involved in transcription control.

Detailed Description of the Invention

Specific targeting of expression of desired genes can be achieved through the selection and use of the regulatory sequences described herein in detail, isolated from the protein C receptor (EPCR). The protein C receptor is the first protein identified and reported with these properties. It is expressed in high levels exclusively in large vessels, and the expression levels decrease with vessel size, until there is little-to-no expression detectable in capillaries.

The EPCR Regulatory Sequences

The endothelial cell protein C binding protein (referred to herein as "EPCR") was cloned and characterized, as described in PCT/US95/09636 "*Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor*" Oklahoma Medical Research Foundation. The protein was predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein C and activated protein C (Kd=30 nM), which is a naturally occurring anticoagulant, and is calcium dependent.

Following identification and cloning of the endothelial cell protein C receptor (EPCR), it was determined that the EPCR was down regulated in cultured endothelial cells by TNFα. To determine the physiological relevance of this finding, EPCR mRNA levels in rats and mice challenged with LD_{95} levels of endotoxin were examined. Surprisingly, in response to endotoxin infusion, EPCR message rose within three hours to about four fold the basal level and remained elevated for twelve hours, then

returning toward baseline at 24 hours. The rapid response suggested that a factor generated by endotoxin infusion could upregulate EPCR expression. Since thrombin is known to be one of these factors, rat microvascular cells in culture were treated with thrombin (0.1 units/ml). The cells exhibited a three to four fold increase in EPCR mRNA levels within three hours relative to control cells.

Physiologically, these results showing elevated mRNA levels three hours after exposure to thrombin, which begins to decline after twelve hours to baseline levels by 24 hours, are important since they suggest that thrombin plays a direct *in vivo* role in upregulation of EPCR expression. High level EPCR expression could contribute to the decrease observed in protein C levels during acute inflammatory response syndromes.

The gene encoding EPCR including the promoter region was then isolated from a murine genomic library, using the DNA encoding murine EPCR as a probe. A human genomic library was similarly screened with the DNA encoding human EPCR to isolate the promoter for the human EPCR Analysis of the promoter revealed a thrombin response element. Gel shift assays revealed that thrombin treatment induced at least one factor that binds specifically to this promoter region. Further analysis yielded the sequence of the thrombin responsive element. This element can be used to increase selective expression in response to thrombin. The promoter is also selective in expression, with the EPCR being selectively expressed more in large vessel endothelial cells when most of the entire promoter is present, including the beginning region. When a shorter portion of the promoter is present, there is expression in all endothelial cells. These results are consistent with a repressor being present in the first part of the promoter which suppresses expression in capillary endothelial cells.

Referring to Figures 1A and 1B and SEQ. ID Nos 1 (the murine EPCR promoter) and 2 (the human EPCR promoter), the 5' regulatory sequences of the EPCR includes a transcription initiation promoter specific to endothelium contained in -1 to -220 (nucleotides 3130 to 3350 of SEQ. ID

No. 1) (referred to for ease of reference as "A"), a control element responsive to thrombin (CCCACCCC) (SEQ. ID No. 3) located between -337 and -345 in the murine promoter (nucleotides 3007 to 3014 of SEQ ID No. 1) and between -360 and -368 in the human promoter (nucleotides 2722 to 2729 of SEQ. ID No. 2) (referred to as "B"), a serum response element located between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1) (referred to as "D"), and a large vessel expression element located between -1080 and -700 (nucleotides 2270 to 2840 of SEQ. ID No. 1) (referred to as "C"). The latter directs expression primarily to large vessels such as aorta, coronary arteries, arteries and veins, rather than to capillaries.

Figures 1A and 1B are a comparison of the sequences from the murine and human promoters, demonstrating that they are highly homologous. It is understood that the equivalent regions from the promoters of EPCR from other species could be used to achieve the same type of expression, and that sequences from different species could be used in combination, for example, A from the murine promoter and C from the human promoter.

Expression Constructs

These regulatory elements can be used alone or in various combinations, as demonstrated by the examples, to determine where and to what extent expression is obtained, both *in vitro* and *in vivo*. Region A can drive endothelial cell specific expression. Adding to this region A, region C would result in expression occurring primarily in large vessels. Adding region B to these regions A and C, results in a thrombin response - i.e., expression is increased by exposure to thrombin, as would occur in a patient during initiation of coagulation or an inflammatory response.

The regulatory sequences can be inserted into vectors for expression using standard recombinant techniques.

The Regulatory Elements are useful as Reagents

The nucleotide sequences are important as hybridization probes, in selected expression of recombinant proteins other than EPCR, in increasing expression of recombinant proteins by exposure of the

encoding construct to thrombin, and in design and screening of drugs and diagnostics for therapeutic and research purposes.

Methods of Treatment

The constructs are particularly useful in gene therapy. The elements can be used to regulate expression of a gene encoding an important protein, or a biologically active nucleic acid molecule such as antisense, triplex forming molecules, ribozymes, and guide sequences for RNAase P which can be used to mutate or stop transcription of a particular gene. Examples of gene targeting include expression of thrombomodulin (TM), EPCR, TFPI, tPA, or heparin (heparan proteoglycans) in large vessel endothelium to decrease clot propensity at atheromas or in autoimmune diseases. If systemic elevations of tPA was desired, sequence A could be used on the gene. Endogenous gene expression could be suppressed by using sequence A, ABC or possibly AC, coupled to antisense to block expression of adhesion molecules to decrease leukocyte infiltration in atherosclerosis. The thrombin response element is significantly inducible in vivo, and should therefore be particularly useful in the treatment of patients with a history of constitutively elevated levels of thrombin, for example, particularly for expression of therapeutic genes in coronary arteries in patients with unstable angina.

The present invention will be further understood by reference to the following non-limiting examples:

Example 1: Isolation of Endothelium and Large Vessel Endothelium specific transcription initiator elements.

Nucleotide sequences were determined for 8.8 kb of the genomic structure and 3.4 kb of the 5'-flanking region of the mouse EPCR (mEPCR) gene. RNase protection assay revealed six major transcription start sites clustered at -110 to -119 upstream of the translation initiation site. A series of 5'-promoter deletion fragments: mP3340, mP1120, mP700, mP350 and an SV40 control were fused to a luciferase reporter gene and transiently transfected into several cell types, bovine aorta

endothelial cells (large vessel endothelial cells), rat heart endothelial cells which is mostly capillary endothelial cells (small vessel endothelial cells), and 293 kidney cells (non-endothelial cells).

The results are shown in Figure 2. The expression was relatively endothelial cell specific.

Deletion of the sequence between -280 to -160 dramatically reduced luciferase expression in bovine aorta cells, as shown by Figure 3. This region of the mEPCR gene (-220 to -180) contains one AP-4 site and two overlapping SP-1 sites, as depicted in Figure 4. Mutations in the core sequence of the AP-4 site and two overlapping SP-1 sites impaired both nuclear protein binding and luciferase expression. These results indicate important roles for AP-4 and SP-1 in the constitutive expression of mEPCR.

Example 2: Thrombin response element.

A thrombin response element (CCCACCCC) (SEQ. ID No. 3) within the upstream region (-337 to -343) was found to mediate the induction of mEPCR by thrombin. In addition, a 380 bp fragment which spans the sequences from -1080 to -700 was identified as the endothelial cell-type specific promoter in cultured cells. This fragment could drive expression of luciferase or green fluorescent protein in large vessel endothelium but not in microvascular or capillary cells, as also shown by Figure 2.

Example 3: *In vivo* Activity of the EPCR Promoter.

Transgenic mice were developed using either the -350 to -1 or -1080 to -1 regions of the mouse EPCR promoter to drive the structural gene for green fluorescent protein (GFP) to determine the *in vivo* activity of the previously described promoter regions.

The promoter regions (-1080 and -350) of mouse EPCR gene were cloned into the pEGFP1 vector (Clontech), which already contains the structural gene for GFP. The fragments which contained the promoter region of mEPCR and GFP reporter gene were released by enzymes Eco47 III and Afl II from the constructs pEGFP350 and pEGFP1080.

After purification, the DNA fragments were microinjected into the pronuclei of fertilized mouse eggs by standard methods. Mice were screened for the presence of the transgene by GFP specific PCR and Southern blotting by standard methods. Several transgenic lines were established from both promoter constructs.

GFP mRNA was constitutively expressed in these lines. The level of GFP mRNA expression was variable from significantly less than to higher than the endogenous EPCR expression. These data indicate that the ability to express a foreign structural gene under the control of these promoters will not be chromosome integration position dependent, although constitutive level of expression may be influenced by chromosomal positioning.

Example 4: LPS Inducibility of the EGFP1080 and EGFP350 constructs in transgenic animals

Animals bearing the EGFP1080 construct and animals bearing the EGFP350 construct were treated with 400 micrograms LPS for 3 hours. Quantitative RT-PCR was performed to determine the level of GFP mRNA present before and after induction. GFP and mEPCR MIMICs (500 bp in length) were prepared by use of the MIMIC construction kit (Clontech). 2 micrograms of total RNA from the mice was used for synthesis of cDNA. Equal sized aliquots were then amplified in the presence of 2 microliters of a 10-fold dilution series of the appropriate MIMIC= (GFP or mEPCR). Equal aliquots were then run on a 2% ethylene bromide agarose gel. The target size is 300 bp and the MIMIC is 500 bp. The ability of the bonafide message to compete for its "MIMIC" at a particular dilution of the MIMIC indicates the abundance of the message in the original sample. Before LPS induction, the GFP mimic could not be effectively competed by the animal's mRNA until the mimic was diluted 1:100.000 for the P1080 animal and 1:106 for the P350 animal. After 3 hr treatment with 400 micrograms LPS, the EGFP1080 animal expressed at least ten times more message (mimic is

effectively competed at a 1:10,000 dilution). The EGFP350 animal could at least partially compete at the same level.

The finding that expression can be induced by treatment of the animals with endotoxin indicates that the response elements are functional *in vivo*, and with heterologous proteins.

Modifications and variations of the methods and materials described herein will be obvious to those skilled in the art from the foregoing detailed description, and are intended to come within the scope of the appended claims. In particular, further definition of the minimal regulatory elements using standard approachs similar to those described herein would be considered obvious equivalents.

SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 3
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(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3360 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(ix) FEATURE:
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(B) SOURCE murine
(D) OTHER INFORMATION: /note= "Nucleotides 2270 through 2840 are a large vessel endothelial specific element".
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) SOURCE murine
(D) OTHER INFORMATION: /note= "Nucleotides 2990 through 3061 are a serum response element".
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) SOURCE murine
(D) OTHER INFORMATION: /note= "Nucleotides 3007 through 3014 are a thrombin response element".
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) SOURCE murine
(D) OTHER INFORMATION: /note= "Nucleotides 3130 through 3350 are an endothelial specific element".
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTATAGCAGC ACCAGACCAG CCTCACACAC ATATACTCAC ACACACACAC ACACACACAC 240

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ACACTGCTGT ATTGTTTAGG ATGCGTGCAT AGATGGTATA GTTATACAGA AATGCAAAGA 420

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CCTGAGGAAC CCGAGCCTGC CCCGACCCAG GTGGGACCCA GAACTCCAGG ATGTTGACGA 3360

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3097 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) SOURCE Human
(D) OTHER INFORMATION: /note= "Nucleotides 2722 through 2729 are a thrombin responsive element".
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTGGAAAAAA ACTTAAGTGT TCAGCAACAA GAGAATGGAT ACATAAATAA TGATCTATTC 60

CCAAAATTGA TTTTTTTTTT TGAGACAGGG TCTTGCTCTG TTGCCCAGGC TAGAGTGCAG 120

TGGCATGATC ATGGCTCACT GCAGTCTCAA CCTCCTGGGC TCAAGCAATC CTCCTACCTC 180

AGCCTCCTGA GTAGCTGAGA CCACAGGCAC AACCCATCAC ACCCAGCTAA TTTTATTTTT 240

TTTGTAGAGA TGGGGGTCTC ACTATGTTGC CCAGGCTGGT CTTGAACTCC TGGGCTCAAA 300

TGATCCACCC ACCTCGGCCT CCAAAGTGCT GGGATAATAC CTCCCCAGCC GGATATTTTA 360

AAGCAGTGAA AATGAATGGT CTACACATAG CCACATGAAT GAATCTTATT AATACATTAA 420

GTGAAAAAAA GCAAAGGTCA CAGAGGAATA CATACATTTT AATACCATTT ATATAAAGCT 480

CAAAATATGT GAAATACCAC TATCTATTGT TTAGGGATAT ATACATAAGT AGTGTAAGTA 540

TACAGAAATA TAAGGAAATG AAAAATATCA AATCTTCATT TTCATCTGAA GTGGTTACTT 600

CAGGGGCTGT GGCAGGGAGA GAGAGATGCA GCTGAGGAAG AGTCCATAGG GGGCTTCAAC 660

TATATTAGCA ATATTGTATT TCTTATGCTT GGTGGTGGGG ATAGGTATGT TTGAAATGTA 720

ATCCTTTAAG CATGAAATAA CTCTTCAAAA ATGAAATATT TCAGGCTGTG CACAGTGGCT 780

CAGGCATTGT AATCCCAGCA TGTTGGGAGG CTGAACGTGG GCGGATCACC GTGAGGTCAG 840

GAGTTTGAGA CCAACCTGGC CAACATGGTG AAATCCCATC TCTACTAAAA ATACAAAAAT 900

TAGCCAGGTG TGGTGGCAGG TGACTGTAAT CCCAGCTACT TGGGAGGCTG AGGCAGGAGA 960

ATCGCTTGAA TCTGGGAGGT GGAGGTTGCA GTGAGCCGAG ATCACGCCAC TGCATTACAG 1020

CAAGACTCCA TCTCAAAAAA AAGAAAAAAA AAAAGAAAAA AGAAATGTTT CATAATTTTT 1080

AATAAAAGGC AAGACAATAT AAATTGGTAG TTATTTAAGT CATTCTACTT TTCCTGAGGC 1140

CCAGTGCAGG AAAACAAAGT TCCTATCCTT GTTCCAACTA GACCATTTTG ATAAGCTGCA 1200

AAAAGAAAAG ACTTTGATGC TATTTCTTAG CCAGTTTGCA ACAGCTGAGA GGTGAGCATG 1260

GAAGCTCTTG CATATATTCA GTTCAGAGAA TGGGTGCTTA GTTTATGTCC AGAGTTTGTC 1320

CCAGATTTCA CTATGACGTC AGCTCTCCGG GGAGAAGTAT ATAAAATAAA AAGTTAAAAT 1380

CCCTCTCAGT CCTTTACCCA ATCCTATTCC CCAGAGGTAA TCTCTATTGA CAGTACCCCT 1440

CCAGATATTT TCCCTATGTA TATACAAATA CACAGATACA CACTGAAAGT TAATTTTGGC 1500

CAGGTGCAGT GGCTCCTGCC TATACCAGAG GATTGCTTGA GTGCAGGAGT TCAAGACCAG 1560

CCTGGGCAAC ATAGCGAGAC CACATCTCTA GTAAAAATAA AAAAAAATAG CTAGGCGTGG 1620

TGGCACAGTG GCACGTACCT TTAGTCTCAG CTACTCGGGT GGTTGAGGTG GAGAATCACT 1680

TGAGCCCGGG GAGGTCAAGC CTACAATTAG CTGTGATTGC TTCACTGCAC TATAGCCTGG 1740

GCAACAGAGC TAGACCCTGT CTCAAAAAAA TAATAATAAA TTTTATATAT ATATATGAGG 1800

ATGAAATTAC ATATGTATTA TTTGAACAGA AGTGAAATCT TTTCTTTTTT TTTTTCAGAC 1860

AGAATCTTGC CGCATGACCC AGGCTAGAAT GCAGTGGTGT GATCTCGGCC CTCTGCAACC 1920

TCCACCTCCC AGGTTCAAGC GATTCTCATG CCTCGGTCTC CCAAGTAGCT GGGATTACAG 1980

GCATGCACCA CCATGCCCAG CTAATTTTTG TATTTTTCGT AGAGACGTTC GCCATATTGG 2040

CCAGGCTGGT CTCAAACTCC TGGCCTCAAG TGATCTGCCC ACCTCGGCCT CCCAAAGTGC 2100

CAGCAGCATG CTCGGAGGAG TGACTTTAAA GCTTTTCTAC TTGCTTCCTA GAGTAAGGGA 2160

CGCATTTTAC ACTGCTATCC AAAACTCATC ATAGAAACAT ACACACACAA AACCAAAGCA 2220

CACATATACA ACTGAGCAAA TATTTCATGA CATAACACTT TCTCTTACTA AGGGTGACGC 2280

GCTGAAATTT TGTATTCTGT CCTATTTCAT TTTTTAAAAA TGGTAACCAT GACCTGCTAA 2340

ATTGATTTCA TTGTCCACTA ATAAATTATG ACCTCAGTTT CAAAAAGATT GCTTTAGGTA 2400

ACCAATCATC TTCTGAGATT TATACAGATT GCTCATAATT CTCTCCTATT TTTTAAAAAC 2460

ATGCTGCAGT GAACTGCTTT ACACTCATTT TATGACTACT TCTGAGACCA AGATCCCGGA 2520

TTATGTAATT GTTATTTACT TAAAATTCTG GTAAAATGTA GCCATTATAC TGGAAAACTA 2580

AATTTTAATC TTGGATCTGT CACCACCATG ATATATAAAC TTTGGGCAAG TCCCTGCACC 2640

TCTCTGGACC TCAATCTCCC CATCAGCAAC CTGCTGATCC TACTCCCAGG AGTGTGCTCT 2700

AAGTTGAAAG TAGATGCCCC ACCCCCTGAG TCAGCGCCGG CAGGACTTCT CACCAAGCCC 2760

TTCTCCCCCT TTTCCGCTCC CTGTTCCTGG TTCCTAGGAA GCAGCCCAAG GAGAAGGGAA 2820

AAGGCAGGTC TGGGCAGGAG GGAGCAATGA AGGGCGGGGC AGAGGGAGGG CAGGAGGGAG 2880

GCCGGCCCCC TAGTAGGAAA TGAGACACAG TAGAAATAAC ACTTTATAAG CCTCTTCCTC 2940

CTCCCATCTC CTGGCCTCCT TCCATCCTCC TCTGCCCAGA CTCCGCCCCT CCCAGACGGT 3000

CCTCACTTCT CTTTTCCCTA GACTGCAGCC AGCGGAGCCC GCAGCCGGCC CGAGCCAGGA 3060

ACCCAGGTCC GGAGCCTCAA CTTCAGGATG TTGACAA 3097

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCACCCC 8

We claim:

1. An isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells.

2. The element of claim 1 depicted in SEQ. ID No. 1, between nucleotides 3130 and 3350.

3. The element of claim 1 depicted in SEQ. ID No. 2.

4. An isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells.

5. The element of claim 1 depicted in SEQ. ID No. 1, between nucleotides 2270 and 2840.

6. The element of claim 1 depicted in SEQ. ID No. 2.

7. An isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum.

8. The element of claim 1 depicted in SEQ. ID No. 1, between nucleotides 2990 and 3061.

9. The element of claim 1 depicted in SEQ. ID No. 2.

10. A construct for expression of a heterologous gene comprising a regulatory element selected from the group consisting of an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells, an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells, and an isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum.

11. The construct of claim 10 further comprising a heterologous gene.

12. The construct of claim 10 further comprising a thrombin response element depicted in SEQ ID No. 3.

13. The construct of claim 10 comprising an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells and an isolated regulatory element

present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells.

14. The construct of claim 13 further comprising the thrombin response element depicted in SEQ ID No. 3.

15. A method for controllably expressing a gene or biologically active nucleotide molecule comprising expressing the gene or biologically active nucleotide molecule under the control of a regulatory element selected from the group consisting of an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells, an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells, and an isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum.

16. The method of claim 15 wherein the gene is also expressed under the control of a thrombin response element depicted in SEQ ID No. 3

17. The method of claim 15 wherein the gene is expressed under the control of an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to endothelial cells and an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to large vessel endothelial cells.

18. The method of claim 15 wherein a gene encoding a protein is expressed.

19. The method of claim 15 wherein a biologically active nucleic acid molecule selected from the group consisting of antisense, triplex forming molecules, ribozymes, and guide sequences for RNAase P, is expressed.

20. The method of claim 15 wherein the gene or biologically active nucleotide molecule is expressed in a patient in need of treatment thereof.

21. The method of claim 15 wherein the gene or biologically active nucleotide molecule is expressed in cell culture.

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2993 TCGAAAGCAGACGGCCCCACCCCCCTGACTCAGCGGCGACCTACCGGACTT 3042
     |  ||||||  |||  |||  |||||||||  ||||||        ||  |  |||||||
2707 TTGAAAGTAGATGCCCCCA.CCCCCCTGAGTCAGCG....CCGGCAGGACTT 2751

3043 CTCGCCAAGCCCCTTCTCCCCCTTTTCCGCTCC.....TCCTCAAGCCTCGG 3088
     |||  |||||||||||||||||||||||||||||      ||||      |||  ||
2752 CTCACCAAGCCCTTCTCCCCCTTTTCCGCTCCCTGTTCCTGGTTCCTAGG 2801

3089 AAGCAAGCAGCGGGAGGAGAAACAGGCAGGTCCAGGCAGGAGGGCCCACA 3138
     |||||  |    ||  | ||  ||||||||||  ||||||||||||  ||
2802 AAGCAGCCCAAGGAGAAGGGAAAAGGCAGGTCTGGGCAGGAGGGAGCAAT 2851

3139 GCTGGGAGGGGC.....CGAGGCGAGCCGGCCCCCTAGTAGGA 3176
     |  |||  ||||||        ||||  ||||||||||||||||||||
2852 GAAGGGCGGGGCAGAGGGAGGGCAGGAGGCCGGCCCCCTAGTAGGA 2901

3177 AATGAGACAGA.TCCAAGTAACACTTTAAAAGCCTGACTCCCTCTTCCTG 3225
     ||||||||||  |  |  ||  ||||||||||  ||||||  |||  ||  ||
2902 AATGAGACACAGTAGAAATAACACTTTATAAGCCT.CTTCCTCCTCCCAT 2950

3226 CACGCGTTCTCTTTCCATCCTCC...GCTCTGCTCCGGCCCCCTCCCGGAC 3272
     |  |  |  |||  |||||||||||  ||  |  |  |  ||||||||||  |||
2951 CTCCTGGCCTCCTTCCATCCTCCTCTGCCCAGACTCCGCCCCCTCCCAGAC 3000
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FIG. 1A

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3273 AG.CCTCCCTTCTCTTT..CCTAATCAGCAGCCTGAGGAACCCGAGCCTG 3319
3001 GGTCCTCACTTCTCTTTTCCCTAGACTGCAGCCAGCGGAGCCCGCAGCCG 3050

3320 CCCCGACCCAGG.........TGGGACCCAGAACTCCAGGATGTTGACGA 3360
3051 GCCCGAGCCAGGAACCCAGGTCCGGAGCCTCAACTTCAGGATGTTGACAA 3100

3361 AGTTTCTGCCGCTACTGCTGCTGCTGCTGCCTGGCTGCGCCCTTTGTA.. 3408
3101 CATTGCTGCCGATA......CTGCTGCTGTCTGGCTGGGCCTTTTGTAGC 3144

3409 .......ACTCCGATGGTGAGTTTGGGTCAAGGCTCCTGCCTGGGGGT.G 3450
3145 CAAGACGCCTCAGATGGTGAGTCGGGGGCACATCTCCTGCCTCAGGATGG 3194

3451 TTCTAGGGA..CTTGGTGTATTTGGAACTTTG 3481
3195 TTCTGGAGAATCTCAGTCTATCTGGCACATGG 3227
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FIG. 1B

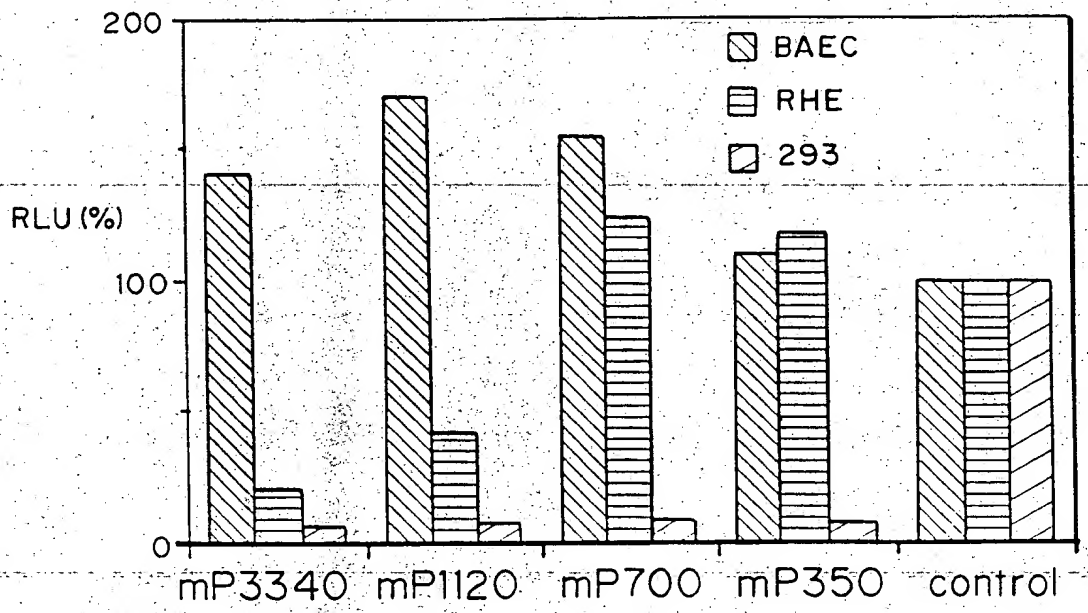
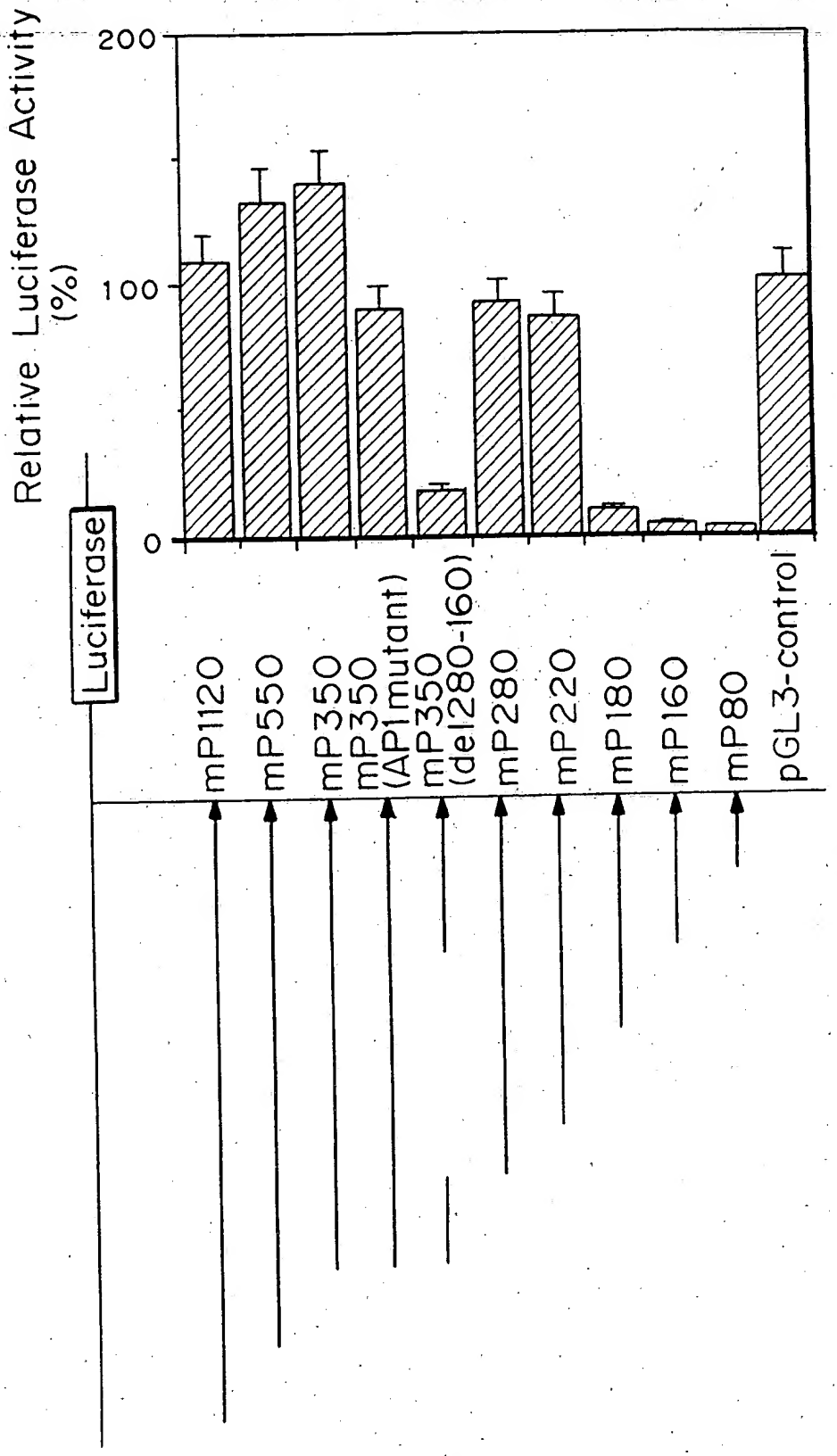


FIG. 2

FIG. 3


Relative Luciferase Activity (%)
200
100
0
Luciferase
mP1120
mP550
mP350
mP350 (AP1mutant)
mP350 (del280-160)
mP280
mP220
mP180
mP160
mP80
pGL3-control

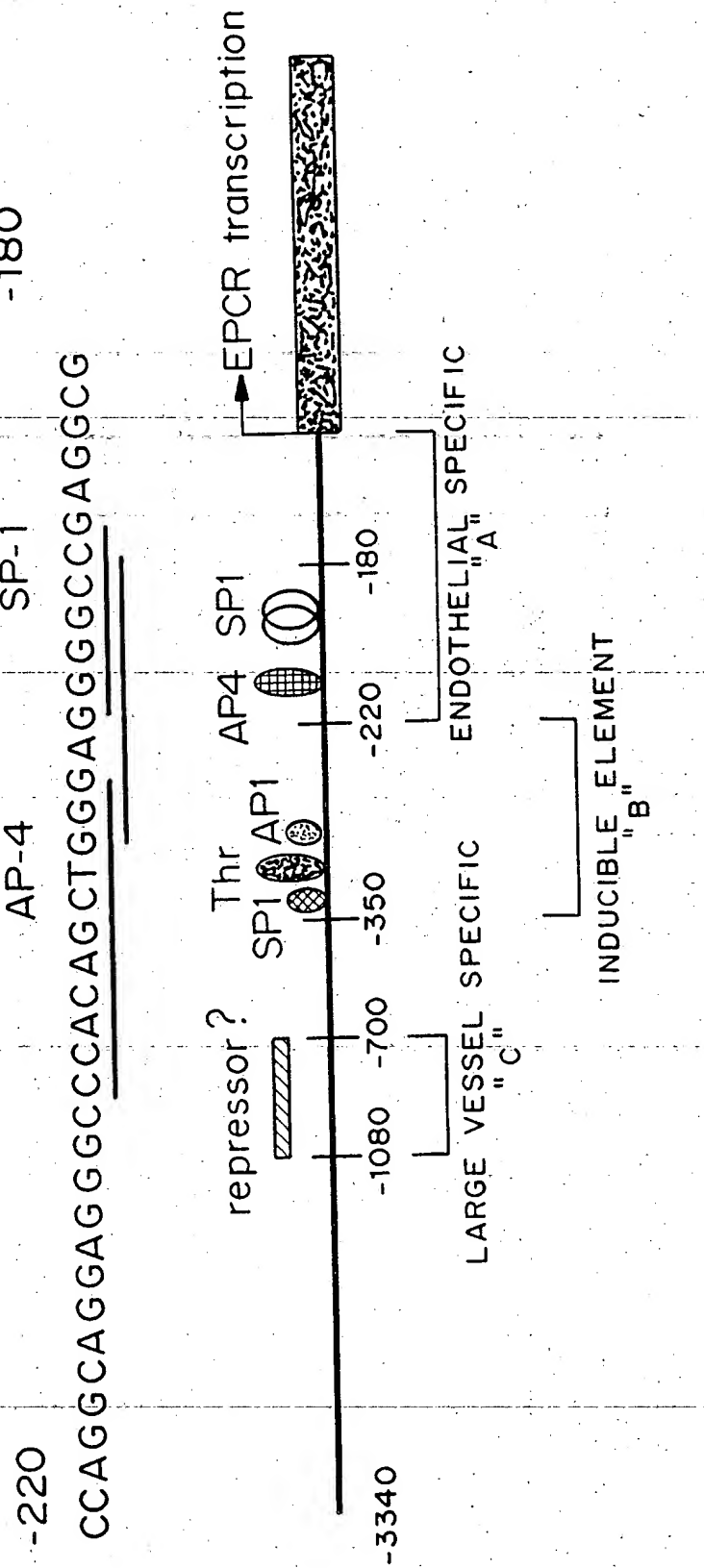

-180
SP-1
AP-4
-220
CCAGGCAGGAGGGCCCACAGCTGGGAGGGGCCGAGGCG
EPCR transcription
AP4 SP1
-180
-220
ENDOTHELIAL SPECIFIC "A"
Thr
AP1
SP1
-350
INDUCIBLE ELEMENT "B"
repressor?
-700
-1080
LARGE VESSEL SPECIFIC "C"
-3340

FIG. 4

INTERNATIONAL SEARCH REPORT

Internat: Application No
PCT/US 97/20364

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. FUKUDOME AND C.T. ESMON: "Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR)" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 10, 1995, BETHESDA, MD, US, pages 5571-5577, XP002058876	1,4
Y	see the whole document and specially figure 1	12,16
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1998	07. 04. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 97/20364

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	W. DING ET AL., : "Upregulation of the message for rodent endothelial cell protein C receptor (EPCR) by endotoxin and thrombin" CIRCULATION, vol. 94, no. suppl. 8, 15 October 1996, NEY YORK, NY, US, page I-694 XP002058206 see abstract	12,16
X	EMBL sequences database, HEIDELBERG, DE M. Marra - 9 August 1996 Mus musculus cDNA clone similar to centrocylin The WasU-HHMI mouse EST project XP002058879 Accession number: AA016910 see abstract	1,2
X	D. SEMON ET AL., : "Nucleotide sequence of the murine TNF locus, including the TNF-alpha (tumor necrosis factor) and TNF-beta (lymphotoxin) genes" NUCLEIC ACIDS RESEARCH, vol. 15, no. 21, 1987, OXFORD, GB, pages 9083-9084, XP002059704 see the whole document	5
X	S.M. GARDNER ET AL., : "Mouse lymphotoxin and tumor necrosis factor: Structural analysis of the cloned genes, physical linkage, and chromosomal position" JOURNAL OF IMMUNOLOGY, vol. 139, no. 2, 1987, BETHESDA, MD, US, pages 476-483, XP002059705 see figure 3	5
X	A. PELERAUX ET AL., : "Genomic organisation of a mouse MHC class II region including the H2-M and Lamp2 loci" IMMUNOGENETICS, vol. 43, 1996, NEW YORK, NY, US, pages 204-214, XP002059706 see figures 3A,B	5
X	WO 96 05303 A (OKLAHOMA MED RES FOUND) 22 February 1996 cited in the application see abstract see page 27, line 13 - page 31, line 21 see page 14, line 6-30	15,16,20

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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 97/20364

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 94 04694 A (MOUNT SINAI HOSPITAL CORP) 3 March 1994 see page 1, line 32 - page 2, line 3 see page 14, paragraph 3 - page 15, paragraph 2	1,4,10, 11,13, 15,17,18
A	WO 93 09236 A (BAYLOR COLLEGE MEDICINE) 13 May 1993 see abstract see page 11, line 27 - page 12, line 5	7,10,15
A	N. BANGALORE ET AL., : "High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells" THROMBOSIS AND HAEMOSTASIS, vol. 72, no. 3, 1994, STUTTGART, DE, pages 465-474, XP002059708 see the whole document	10,11,15
P,X	EMBL sequences database, HEIDELBERG, DE M. Marra - 25 July 1997 Mus musculus cDNA similar to M. musculus endothelial cell activated protein C receptor. The WashU-HHMI mouse EST project XP002058878 accession number: AA389039 see abstract	1,2
P,X	EMBL sequences database, HEIDELBERG, DE M. Marra - 14 December 1996 Mus musculus cDNA clone The WashU-HHMI mouse EST project XP002058880 Accession number: AA146223 see abstract	4,5
T	J.M. GU AND C.T. ESMON: "Functional characterization of the 5'-flanking region of the mouse endothelial protein C receptor (EPCR) gene" MOLECULAR BIOLOGY OF THE CELL, no. suppl. 8, 15 December 1997, BETHESDA, MD, US, page 228a XP002058877 see abstract	1,4,11, 13,17, 18,21

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna. Application No
PCT/US 97/20364

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9605303 A	22-02-96	US 5695993 A	09-12-97
		AU 3272395 A	07-03-96
		CA 2199821 A	22-02-96
		EP 0777731 A	11-06-97
WO 9404694 A	03-03-94	US 5466596 A	14-11-95
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		EP 0658209 A	21-06-95
		JP 8500247 T	16-01-96
WO 9309236 A	13-05-93	US 5298422 A	29-03-94
		AU 660751 B	06-07-95
		AU 3124693 A	07-06-93
		CA 2122617 A	13-05-93
		EP 0635060 A	25-01-95
		JP 7500967 T	02-02-95
		PT 101042 A	28-02-94